

PATENT APPLICATION

TENEBRIO ANTIFREEZE PROTEINS

Inventors: Laurie A. Graham, of 31 Stanley Street, Apt. 13,
Kingston, Ontario, CANADA K7K 1X8
a citizen of Canada

Yih-Cherng Liou, of 15 MacPherson Avenue, Apt. 18-102,
Kingston, Ontario, CANADA K7M 2W8
a citizen of Canada

Virginia K. Walker, of R.R. #1,
Sydenham, Ontario, CANADA K0H 2T0
a citizen of Canada

Peter L. Davies, of 100 Dickens Drive,
Kingston, Ontario, CANADA K7M 2M8
a citizen of Canada

Assignee: Queen's University at Kingston
Kingston, Ontario
Canada

TOWNSEND and TOWNSEND and CREW, LLP
Two Embarcadero Center, 8th Floor
San Francisco, California 94111-3834
(415) 576-0200

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TENEbrio ANTIFREEZE PROTEINS

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Not applicable.

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For biologics, such as therapeutic drugs, blood plasma, mammalian cells for use in tissue culture, and the like, freezing can cause extensive damage. For example, the freezing process itself kills most eukaryotic cells, and cells subjected to even one freezing and thawing cycle exhibit greatly reduced viability. Impaired function of living cells is also prevalent in tissue cryopreservation, with concomitant drawbacks for organ transplants. Similarly, frost or other freezing damage to plants presents a serious problem in agriculture. Finally, drugs can become ineffective, or even dangerous, if not maintained under required strict temperature conditions.

Although the first description of protein-mediated thermal hysteresis (TH, as defined below) was noted in *Tenebrio molitor* hemolymph approximately 30 years ago (Grimstone, *et al.*, *Philos. Trans. B* **253**:343 (1968)), numerous attempts to purify these thermal hysteresis proteins (THP) failed to yield pure fractions with enough TH to account for the hemolymph activity (Grimstone, *et al.*, (1968); Paterson & Duman, *J. Exp. Zool.* **210**:361 (1979); Schneppenheim & Theede *Comp. Biochem. Physiol.* **67B**:561 (1980); Tomchaney, *et al.*, *Biochemistry* **21**:716 (1982); Paterson & Duman *J. Exp. Zool.* **219**:381 (1982); and (Horwath, *et al.*, *Eur. J. Entomol.* **93**: 419 (1996)). Homogeneity of these proteins was not proven, and they differed in amino acid composition from each other and from the compositions reported here.

There exists a need for new techniques and compositions suitable for improving the preservation characteristics of organic materials at low temperatures, including storage of frozen foods and the viability of biologics. Ideally, these techniques and compositions will be inexpensive, yet completely safe and suitable for human consumption or *in vivo* therapeutic uses. There also exists a need for new techniques and compositions suitable for depressing the freezing point or inhibiting freezing in non-organic systems, such as in deicing treatments. The present invention fulfills these and other needs.

SUMMARY OF THE INVENTION

The common yellow mealworm beetle, *Tenebrio molitor*, is a freeze-tolerant pest of stored grains in temperate regions. Larvae are able to supercool to an average temperature of -12°C (Johnston & Lee, *Cryobiol.* **27**:562 (1990)). A second line of defense against freezing is the thermal hysteresis (TH) activity of their hemolymph, which

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The invention provides for an organism into which, or into an ancestor thereof, an exogenous nucleic acid sequence which specifically hybridizes under stringent conditions to SEQ ID NO: 2 or 5 or the nucleic acid of claim 1 has been introduced, and

the organism translates the exogenous nucleic acid into an antifreeze protein. Also provided for is an organism with an exogenous nucleic acid sequence which is translated into an antifreeze protein that is expressed externally from the organism. In a preferred embodiment, the organism is a fish. In further preferred embodiments, the organism is a fish is kept in a salt-water environment, or, the fish is a member of the family *Salmonidae*. In other preferred embodiments, the organism can be a plant, a fungus, a yeast or a bacteria. In another embodiment, if the organism is a yeast, it can be selected from the group consisting of *Torulopsis holmii*, *Saccharomyces fragilis*, *Saccharomyces cerevisiae*, *Saccharomyces lactis*, and *Candida pseudotropicalis*. In another embodiment, if the organism is a bacterium, it can be selected from the group consisting of *Escherichia coli*, *Streptococcus cremoris*, *Streptococcus lactis*, *Streptococcus thermophilus*, *Leuconostoc citrovorum*, *Leuconostoc mesenteroides*, *Lactobacillus acidophilus*, *Lactobacillus lactis*, *Bifidobacterium bifidum*, *Bifidobacterium breve*, and *Bifidobacterium longum*. Plants transformed with THP sequences can include grapes, oilseed plants such as canola, grains, citrus and sugar cane.

The invention provides for a method for decreasing the freezing point of an aqueous solution involving the addition of an antifreeze protein to the aqueous solution. In a preferred embodiment, the method involves the addition of the antifreeze protein encoded by the nucleic acid of claim 1 to the aqueous solution. In other preferred embodiments, the aqueous solution is applied to an organism; the antifreeze protein is produced by recombinant means; the antifreeze protein can specifically bind to the antibody of claim 13 or claim 14, the antifreeze protein is selected from the group consisting of YL-1, YL-2, YL-3 and YL-4; and/or, the antifreeze protein is encoded by a nucleic acid molecule which specifically hybridizes to the nucleic acid of SEQ ID NO:2 or 5.

In addition, it is contemplated that the addition of the THP of this invention to aqueous solutions may better preserve organs and other biologicals in transit.

A further understanding of the nature and advantages of the present invention may be realized by reference to the remaining portions of the specification, the figures and claims.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 is a chromatogram of diluted *Tenebrio* hemolymph loaded onto an S-100 SEPHACRYL® (Pharmacia) column (92 cm x 1.6 cm) and eluted with hemolymph buffer without phenylthiocarbamide (see text).

Figure 2 is a chromatogram of selected active fractions from gel exclusion chromatography which were combined and chromatographed by reversed-phase HPLC on a C18 analytical column (Vidac), using a gradient of 0.4% acetonitrile/min in 0.05% trifluoroacetic acid.

Figure 3 is a 15% SDS-PAGE of reverse-phase HPLC fractions which were lyophilized and resuspended in 50 µL of 0.1 M NH₄HCO₃ (pH 8.0). The gel was stained using the Silver Stain Plus Kit (Bio-Rad). Approximate molecular weights were determined by MALDI mass spectrometry.

Figure 4 is a graph indicating the hyperbolic nature of the thermal hysteresis activity of *Tenebrio* hemolymph.

Figures 5 I-IV, are micro-photographs of ice crystals grown in the presence of *Tenebrio* THP (I and II) and fish antifreeze proteins (III and IV).

Figure 6 is an alignment chart of recombinant isoforms of THP. The positions in which the nucleotide is conserved in all cDNA sequences are marked by an asterisk (*). The complete amino acid sequence is indicated only for YL-1. Residues of other isoforms which are identical to those found in YL-1 are indicated by a period (.) Differences are shown where found. Gaps in both the cDNA and protein sequences are indicated by dashes (-----).

DETAILED DESCRIPTION OF THE INVENTION

This invention relates to isolated nucleic acid sequences encoding a novel class of antifreeze protein (THP). The procedure for obtaining natural THP genes generally involves constructing or obtaining a gene library from *Tenebrio molitor*, detecting and isolating the desired gene, cloning it, and expressing it in a suitable host cell and then purifying the expressed protein. The natural protein can be used without modification or it can be modified in a variety of ways without affecting its TH activity.

The amino acid compositions of the purified proteins and the deduced mature sequences are very similar. The THPs are particularly rich in Cys (18-19%) and Thr (20-26%) and deficient in several hydrophobic amino acids, notably Leu and Ile. The overall hydrophilicity is approximately 55% (Wishard, *et al.*, *Comput. Appl. Biosci.* **10**:121 (1994)), which is much higher than that found in fish antifreeze proteins (AFPs) (Sönnichsen, *et al.*, *Prot. Sci.* **4**:460 (1995)).

The primary structure of the mature THP is very unusual (Fig. 4) and is not similar to any other known sequence. The first 21 amino acids contain 6 Cys spaced at irregular intervals (Cx₁Cx₂Cx₃Cx₂Cx₂C; SEQ ID NO:3), and this sequence overlaps with the first of a series of 12-amino-acid repeats that continue until the end of the protein. Cys is repeated at 6-residue intervals throughout this region, which has the consensus sequence CTxSxxCxxAxT (SEQ ID NO:1). The N-terminal Cys spacing has some elements in common with zinc-binding motifs (Klug & Schwabe, *FASEB J.* **9**:597 (1995)). However, extensive dialysis against 10 mM EDTA or 10 mM phenanthroline, and the subsequent addition of 2 mM ZnCl₂ (or 2 mM CaCl₂) to chelator-free preparations incubated for 1 h at 22°C does not affect activity, suggesting that there is no role for divalent metal ions in TH activity. At least some of the Cys residues are involved in disulfide bridges because all activity is lost on incubation with 10 mM dithiothreitol at 37°C for 20 min, whereas no activity is lost under the same conditions in the absence of reducing agent. There is no effect of N-ethylmaleimide on TH activity, which suggests that if free Cys are present they can be modified without loss of activity.

The differences in length between the THP variants represent multiples of the 12-amino-acid repeat, suggesting that each repeat may form a functional domain. Although the THP repeats are short, they may fold independently to form a chain, which could explain the discrepancy between the actual and apparent molecular weights (see, *infra*) of the proteins as being due to asymmetry.

There is no structural similarity between *Tenebrio* THPs and fish AFPs. Fish type II AFP, which contains 10 Cys, does not display any repetitive structure or regularity in Cys spacing. Fish type I AFP and the antifreeze glycoprotein are built up of repetitive elements, but neither contains Cys (Davies & Hew, *FASEB J.* **4**:2460 (1990)). It is, however, intriguing that the most abundant residue in *Tenebrio* THPs, Thr, is also the

amino acid thought to play a central role in binding fish AFPs types I and III to the ice surface (Wen & Laursen *J. Biol. Chem.* 267:14102 (1992); and Chao, *et al.*, *Prot. Sci.* 3:1760 (1994)).

However, like fish antifreeze proteins, *Tenebrio* THP appears to act by an adsorption-inhibition mechanism. Ice crystals in the presence of sufficient THP to produce about 1°C of thermal hysteresis, stop growing until the non-equilibrium freezing point is exceeded. At that point, ice crystals burst forth from the crystal nucleator to form a solid mass of ice along the α -axis. At low thermal hysteresis activities, the ice fronts are broad and smooth. However, at high thermal hysteresis values, the ice fronts are no longer smooth. In contrast, once the freezing point is exceeded in the presence of fish AFPs, myriad ice spicules burst out along and parallel to the c -axis.

In addition, similar to fish AFP (DeVries, *Ann. Rev. Physiol.* 45:245 (1983), the relationship between TH activity and THP concentration is hyperbolic. However, the ice crystals formed in the presence of THP are unusual in that they have a pronounced curvature of their surfaces. In contrast, ice crystals generated by fish AFP Types I and III are hexagonal bipyramids with flat, well-defined facets.

The purified, expressed THP protein can be directly added to an aqueous solution to depress the freezing point or in another embodiment, transformed organisms that express the antifreeze proteins can be added to items which will be stored frozen, such as frozen desserts. In yet another embodiment, the transformed organisms, *e.g.*, fish, plants and yeast, need not express the THP proteins extracellularly but the presence of a THP gene and intracellular protein confers to them the increased ability to survive freezing temperatures. For example, a transformed organism can be a saltwater fish. Transformed salt-water fish can include members of the family *Salmonidae*, halibut, sablefish or any edible saltwater species not having any or sufficient levels of antifreeze proteins. Plants transformed with THP sequences can include grapes, oilseed plants such as canola, grains, citrus and sugar cane.

All publications, patents and patent applications cited herein are hereby expressly incorporated by reference for all purposes.

I. DEFINITIONS

The term "antibody" refers to a polypeptide substantially encoded by an immunoglobulin gene or immunoglobulin genes, or fragments thereof, which specifically binds to and recognizes an analyte (antigen). The recognized immunoglobulin genes include the kappa, lambda, alpha, gamma, delta, epsilon and mu constant region genes, as well as the myriad immunoglobulin variable region genes. Light chains are classified as either kappa or lambda. Heavy chains are classified as gamma, mu, alpha, delta, or epsilon, which in turn define the immunoglobulin classes, IgG, IgM, IgA, IgD and IgE, respectively.

An exemplary immunoglobulin (antibody) structural unit comprises a tetramer. Each tetramer is composed of two identical pairs of polypeptide chains, each pair having one "light" (about 25 kD) and one "heavy" chain (about 50-70 kD). The N-terminus of each chain defines a variable region of about 100 to 110 or more amino acids primarily responsible for antigen recognition. The terms variable light chain (V_L) and variable heavy chain (V_H) refer to these light and heavy chains respectively.

Antibodies exist *e.g.*, as intact immunoglobulins or as a number of well characterized fragments produced by digestion with various peptidases. Thus, for example, pepsin digests an antibody below the disulfide linkages in the hinge region to produce $F(ab)_2$, a dimer of Fab which itself is a light chain joined to V_H-C_H1 by a disulfide bond. The $F(ab)_2$ may be reduced under mild conditions to break the disulfide linkage in the hinge region, thereby converting the $F(ab)_2$ dimer into a Fab' monomer. The Fab' monomer is essentially a Fab with part of the hinge region (*see*, FUNDAMENTAL IMMUNOLOGY, 3RD ED., W.E. Paul, ed., Raven Press, N.Y. (1993)). While various antibody fragments are defined in terms of the digestion of an intact antibody, one of skill will appreciate that such fragments may be synthesized *de novo* either chemically or by utilizing recombinant DNA methodology. Thus, the term antibody, as used herein, also includes antibody fragments either produced by the modification of whole antibodies or those synthesized *de novo* using recombinant DNA methodologies (*e.g.*, single chain Fv).

An "antifreeze protein antibody" is an antibody or antibody fragment that specifically binds an antifreeze protein of this invention or a subsequence thereof.

The term "antifreeze protein" refers to a protein found in the body fluids of some poikilothermic organisms, such as the *Tenebrio molitor* mealworm and plants, which have the commonly known property that they reduced non-colligatively the freezing point of water. Antifreeze proteins are also known as "thermal hysteresis proteins" (THPs). As used herein, "antifreeze proteins" or "THPs" includes chemically synthesized and recombinantly produced polypeptides having a protein sequence with substantial similarity to a naturally occurring antifreeze protein and retaining the properties of a natural antifreeze protein.

The phrase "consensus sequence" refers to a nucleic acid or amino acid sequence in which each position represents the residue or base most often found when many actual sequences are compared. SEQ ID NO:1 is a consensus sequence of a repeating motif found in the proteins of the instant invention

The phrase "conserved amino acids" refers to common amino acid residues in two or more appropriately aligned THP-motif sequences. The conserved amino acid sequences can be intrapeptide or interpeptide. For example, in the THPs of the present invention, the amino acid motifs may consist of 5 out of 6 conserved amino acids, or in other words, 5 out of 6 amino acid residues of any particular motif will be common with 5 out of 6 amino acids of the motif either within a THP amino acid sequence or within two or more THP amino acid sequences.

The term "contiguous amino acid motif" refers to a repeating pattern of amino acids present in a polypeptide or protein. The amino acids in each repeat do not have to be the same but there should be a pattern common to all. For example, in the class of proteins of the present invention, the repeating amino acid motif, cys-thr-xaa-ser-xaa-xaa cys-xaa-xaa-ala-xaa-thr, where xaa is any amino acid, is present.

The term "crustacean" refers to the common definition of the word, i.e., a chiefly aquatic arthropod of the class Crustacea, including lobsters, shrimps, crabs and barnacles.

The term "decreasing the freezing point of an aqueous solution" refers to lowering the temperature of an aqueous solution at which ice crystals form and grow. The decrease in freezing point depends both on the agent used to decrease the freezing point and its concentration in the aqueous solution. The freezing point depression increases as

the antifreeze component is added to the aqueous solution, until a maximum depression is observed at a characteristic concentration. Further addition of antifreeze chemicals, such as ethylene glycol, to aqueous solutions will either result in insolubility of the antifreeze composition or serve to increase the freezing point of the mixture. On the other hand, the increase in thermal hysteresis with increased THP concentration is not linear but hyperbolic. The incremental increases in TH that result from unit increases in THP concentration become smaller and smaller as the saturation point of the THP solubility is approached.

The freezing point of a solution with a THP protein is defined as the temperature at which the sample being measured, which contains an ice crystal nucleator, becomes a solid mass of ice. The ice crystals can form spontaneously or expand from an ice nucleator. Spontaneous formation of a solid mass of ice without a nucleator is typically termed the “supercooling ability” of the THP. Because of the absence of an ice nucleator to initiate the ice formation process, supercooling can occur at much lower temperatures.

In addition to inspecting visually ice crystal formation, a thermal hysteresis assay can measure the difference between the freezing and melting points of a solution. The melting point of a solution is the temperature at which there is only one ice crystal left in a solution (see, *infra*, for a more complete description of TH activity).

The phrase “expressed externally” in the context of a recombinant protein refers to the ability of the transformed cell to synthesize and direct the protein into the extracellular matrix. The extracellular matrix can be the interstitial space between cells in a multicellular organism, bacterial broth or tissue culture medium. With bacteria, external expression can also include expression of the recombinant protein into the periplasm of the bacteria. External expression can be by any means, *e.g.*, secretory and transport vesicles, expression as a membrane protein, expression of a cleavable signal peptide, *etc*

The terms “homology,” “sequence identity” and “sequence similarity” in the context of this invention mean that two peptide sequences, when optimally aligned, such as by the programs GAP or BESTFIT using default gap weights, share at least 40 percent sequence identity, preferably at least 50 percent sequence identity, and most preferably at least 60 percent sequence identity. “Percentage amino acid sequence identity” refers to a comparison of the amino acid sequences of two polypeptides which, when optimally aligned, have approximately the designated percentage of the same amino acids. For

example, "60% sequence identity" and "60% homology" refer to a comparison of the amino acid sequences of two polypeptides which, when optimally aligned, have 60% amino acid identity. Preferably, residue positions which are not identical differ by conservative amino acid substitutions. For example, the substitution of amino acids having similar chemical properties such as charge or polarity is not likely to affect the properties of a protein. Examples include glutamine for asparagine or glutamic acid for aspartic acid.

The term "immunologically reactive conditions" refers to an environment in which antibodies can bind to antigens. Typically, this is an immunological binding assay.

The term "isolated," when applied to a nucleic acid or protein, denotes that the nucleic acid or protein is essentially free of other cellular components with which it is associated in the natural state. It is preferably in a homogeneous state, although it can be in either a dry or aqueous solution. Purity and homogeneity are typically determined using analytical chemistry techniques such as polyacrylamide gel electrophoresis (PAGE) or high performance liquid chromatography (HPLC). A protein which is the predominant species present in a preparation is substantially purified.

In particular, an isolated THP gene is separated from open reading frames which naturally flank the gene and encode proteins other than the THP. The term "purified" denotes that a nucleic acid or protein gives rise to essentially one band in an electrophoretic gel. Particularly, it means that the nucleic acid or protein is at least 85% pure, more preferably at least 95% pure, and most preferably at least 99% pure.

The term "nucleic acid molecule" or "nucleic acid sequence" refers to deoxyribonucleotides or ribonucleotides and polymers thereof in either single- or double-stranded form. Unless specifically limited, the term encompasses nucleic acids containing known analogues of natural nucleotides which have similar binding properties as the reference nucleic acid and are metabolized in a manner similar to naturally occurring nucleotides. Unless otherwise indicated, a particular nucleic acid sequence also implicitly encompasses conservatively modified variants thereof (*e.g.* degenerate codon substitutions) and complementary sequences and as well as the sequence explicitly indicated. Specifically, degenerate codon substitutions may be achieved by generating sequences in which the third position of one or more selected (or all) codons is substituted with mixed-base and/or deoxyinosine residues (Batzer *et al.*, *Nucleic Acid Res.* **19**:5081 (1991), Ohtsuka *et al.*, *J.*

The term nucleic acid is used interchangeably with gene, cDNA, and mRNA encoded by a gene.

The phrase "exogenous nucleic acid" generally denotes a nucleic acid that has been isolated, cloned and ligated or chemically synthesized and ligated to a nucleic acid with which it is not combined in nature, and/or introduced into and/or expressed in a cell or cellular environment other than the cell or cellular environment in which said nucleic acid or protein may typically be found in nature. The term encompasses both nucleic acids originally obtained from a different organism or cell type than the cell type in which it is expressed, and also nucleic acids that are obtained from the same cell line as the cell line in which it is expressed.

The phrase "a nucleic acid sequence encoding" refers to a nucleic acid which contains sequence information for a structural RNA such as rRNA, or tRNA, or for mRNA which encodes the primary amino acid sequence of a specific protein or peptide, or a binding site for a trans-acting regulatory agent. This phrase specifically encompasses degenerate codons (*i.e.*, different codons which encode a single amino acid) of the native sequence or sequences which may be introduced to conform with codon preference in a specific host cell.

The term “recombinant means” refers to techniques where proteins are isolated, wherein the cDNA sequence coding the protein is identified and inserted into an expression vector. The vector is then introduced into a cell and the cell expresses the protein. Recombinant means also encompasses the ligation of coding or promoter DNA from different sources into one vector for expression of a fusion protein, constitutive expression of a protein, or inducible expression of a protein.

The phrase "specifically or selectively binds to an antibody" or "specifically immunoreactive with", when referring to a protein or peptide, refers to a binding reaction which is determinative of the presence of the protein in the presence of a heterogeneous population of proteins and other biologics. Thus, under designated immunoassay conditions, the specified antibodies bind to a particular protein and do not bind in a significant amount to other proteins present in the sample. Specific binding to an antibody under such conditions may require an antibody that is selected for its specificity for a

particular protein. For example, antibodies raised against the THP of this invention or to the partially encoded sequence depicted in SEQ ID NO: 4 can be selected to specifically immunoreact with full length protein and not with other proteins except perhaps to polymorphic variants. As described below, a variety of immunoassay formats may be used to select antibodies specifically immunoreactive with a particular protein. For example, solid-phase ELISA immunoassays are routinely used to select monoclonal antibodies specifically immunoreactive with a protein. See Harlow & Lane (1988) ANTIBODIES, A LABORATORY MANUAL, Cold Spring Harbor Publications, New York ("Harlow & Lane"), for a description of immunoassay formats and conditions that can be used to determine specific immunoreactivity. Typically a specific or selective reaction will be at least twice background signal or noise and more typically more than 10 to 100 times background.

The term "specifically hybridizing" refers to a nucleic acid probe that hybridizes, duplexes or binds to a particular target DNA or RNA sequence when the target sequences are present in a preparation of total cellular DNA or RNA. "Complementary" or "target" nucleic acid sequences refer to those nucleic acid sequences which selectively hybridize to a nucleic acid probe. Proper annealing conditions depend, for example, upon a probe's length, base composition, and the number of mismatches and their position on the probe, and must often be determined empirically. For discussions of nucleic acid probe design and annealing conditions, see, for example, Sambrook or CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, Ausubel *et al.*, ed. Greene Publishing and Wiley-Interscience, New York (1987) ("Ausubel").

"Stringent hybridization" and "stringent hybridization wash conditions" in the context of nucleic acid hybridization experiments such as Southern and northern hybridizations are sequence dependent, and are different under different experimental parameters. An extensive guide to the hybridization of nucleic acids is found in Tijssen (1993) LABORATORY TECHNIQUES IN BIOCHEMISTRY AND MOLECULAR BIOLOGY--HYBRIDIZATION WITH NUCLEIC ACID PROBES part I chapter 2 "Overview Of Principles Of Hybridization And The Strategy Of Nucleic Acid Probe Assays", Elsevier, New York. Generally, highly stringent hybridization and wash conditions are selected to be about 5°C lower than the thermal melting point (T_m) for the specific sequence at a defined ionic strength and pH. The T_m is the temperature, under defined ionic strength and pH, at which

50% of the target sequence hybridizes to a perfectly matched probe. Very stringent conditions are selected to be equal to the T_m for a particular probe. An example of stringent hybridization conditions for hybridization of complementary nucleic acids which have more than 100 complementary residues on a filter in a Southern or northern blot is 50% formamide with heparin at 42°C, with the hybridization being carried out overnight. An example of highly stringent wash conditions is 0.15 M NaCl at 72°C for about 15 minutes. An example of stringent wash conditions is a 0.2x SSC wash at 65°C for 15 minutes (*see*, Sambrook, *supra* for a description of SSC buffer). Often, a high stringency wash is preceded by a low stringency wash to remove background probe signal. An example medium stringency wash for a duplex of, *e.g.*, more than 100 nucleotides, is 1x SSC at 45°C for 15 minutes. An example low stringency wash for a duplex of, *e.g.*, more than 100 nucleotides, is 4-6x SSC at 40°C for 15 minutes. In general, a signal to noise ratio of 2x (or higher) than that observed for an unrelated probe in the particular hybridization assay indicates detection of a specific hybridization. Nucleic acids which do not hybridize to each other under stringent conditions are still substantially identical if the polypeptides which they encode are substantially identical. This occurs, *e.g.*, when a copy of a nucleic acid is created using the maximum codon degeneracy permitted by the genetic code.

The term "thermal hysteresis activity" or "TH activity" refers to the ability to alter the temperature difference (°C) between the freezing and melting points of a solution containing ice. Preferably, TH activity is measured by observation of ice crystal formation in a nanoliter osmometer following the procedure set forth in Lawson & Semler, *Proc. Nat'l Acad. Sci. USA* 88:9919 (1991). Alternatively, TH activity can be determined according to the method described in deVries, *METHODS IN ENZYMOLOGY*, VOL. 127, Packer (ed.), Academic Press, New York (1986) or a variation thereof.

For example, in the present invention, the TH activity of the native *Tenebrio molitor* THP from hemolymph at approximately 1 mg/mL is greater than approximately 5°C. The TH activity of the recombinant THP of this invention at approximately 1 mg/mL is about 5°C or less. More preferably, the TH activity is lower than about 3°C. Most preferably, the TH activity is between 1.5 and 3°C. It is hypothesized that the differences between the TH activity of the native THP and the recombinant THP is due to imperfect

folding of at least a portion of the THP of the bacteria-derived recombinant proteins. When properly folded, it is expected that the TH activities of the recombinant THP will be similar to that of the native THP.

II. NUCLEIC ACIDS ENCODING THP

A. General Techniques

The nucleic acid compositions of this invention, whether RNA, cDNA, genomic DNA, or a hybrid of genetic recombinations, may be isolated from natural sources or may be synthesized *in vitro*. The nucleic acids claimed may be present in transformed cells, in a transformed cell lysate, or in a partially purified or substantially pure form.

Techniques for nucleic acid manipulation of genes encoding the antifreeze protein such as generating libraries, subcloning into expression vectors, labeling probes, DNA hybridization, and the like are described generally in Sambrook *et al.*, MOLECULAR CLONING: A LABORATORY MANUAL (2ND ED.), Vols. 1-3, Cold Spring Harbor Laboratory, N.Y. (1989) ("Sambrook"), which is incorporated herein by reference.

Nucleic acids and proteins are detected and quantified herein by any of a number of means well known to those of skill in the art. These include analytical biochemical methods such as spectrophotometry, radiography, electrophoresis, capillary electrophoresis, high performance liquid chromatography (HPLC), thin layer chromatography (TLC), hyperdiffusion chromatography, and the like, and various immunological methods such as fluid or gel precipitin reactions, immunodiffusion (single or double), immunoelectrophoresis, radioimmunoassays (RIAs), enzyme-linked immunosorbent assays (ELISAs), immunofluorescent assays, and the like. The detection of nucleic acids proceeds by well known methods such as Southern analysis, northern analysis, gel electrophoresis, PCR®, radiolabeling, scintillation counting, and affinity chromatography.

B. Isolation of Nucleic Acids Encoding THP

Methods of isolating total DNA or mRNA are well known to those of skill in the art. For example, methods of isolation and purification of nucleic acids are described

in detail in Chapter 3 of LABORATORY TECHNIQUES IN BIOCHEMISTRY AND MOLECULAR BIOLOGY. HYBRIDIZATION WITH NUCLEIC ACID PROBES, *Part I. Theory and Nucleic Acid Preparation*, P. Tijssen, ed. Elsevier, N.Y. (1993) ("Tijssen")

1. Preparation and Screening of DNA Libraries

There are numerous methods for isolating the DNA sequences encoding the antifreeze protein of this invention. For example, DNA may be isolated from a genomic or cDNA library using labeled oligonucleotide probes having sequences complementary to the sequences or subsequences disclosed herein (SEQ ID NOs:2 or 5). Such probes can be used directly in hybridization assays to isolate DNA encoding THP isoforms. Alternatively probes can be designed for use in amplification techniques such as PCR, and DNA encoding THP may be isolated by using methods such as PCR (see *infra*).

To prepare a cDNA library, mRNA is isolated from *Tenebrio* larvae (Carolina Biological Supply, or a local pet or bait shop) or from embryonic, larval or possibly adult cells grown in tissue culture. cDNA is reverse transcribed from the mRNA according to procedures well known in the art and inserted into vectors. The vectors are transfected into a recombinant host for propagation, screening and cloning. Methods for making and screening cDNA libraries are well known. See Gubler & Hoffman, *Gene* 25:263 (1983) and Sambrook, *et al.*

To make a genomic library, total DNA is extracted from the cells of the insect by well-known methods (see Sambrook, *et al.*) and then mechanically sheared or enzymatically digested to yield DNA fragments (*e.g.*, of about 12-20kb). The fragments are then separated by gradient centrifugation from undesired sizes and are inserted in bacteriophage- λ or other vectors. These vectors (*e.g.*, phage) are packaged *in vitro*, as described in Sambrook, *et al.* Recombinant phage are analyzed by plaque hybridization as described in Benton & Davis, *Science*, 196:180 (1977). Colony hybridization is carried out as generally described in Grunstein, *et al.*, *Proc. Natl. Acad. Sci. USA.*, 72:3961 (1975).

DNA encoding THP is identified in either cDNA or genomic libraries by the ability to hybridize with nucleic acid probes, for example SEQ ID NO:2 or 5. Once identified, these DNA regions are isolated by standard methods familiar to those of skill in

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carried out using the two primers (see, Innis). In the instant invention, because of the presence of repetitive motifs, the length of the THP subsequence encoded by the amplified product will depend on the template used. Because the N and C termini are unique (*i.e.*, different nucleotide sequences from the repetitive motif), to amplify the full-length THP encoding sequence, the primers of SEQ ID NOs: 6 and 7 can be used.

PCR can be used in a variety of protocols to isolate nucleic acids encoding partial sequences of THP. In these protocols, appropriate primers and probes for amplifying DNA encoding partial sequences of THPs are generated from analysis of the DNA sequences listed herein. For example, the oligonucleotides of SEQ ID NOs:6, 7, 8 and 9 can be used in a PCR protocol to amplify regions of DNA which encodes THPs. Once such regions are PCR-amplified, they can be sequenced and labeled oligonucleotide probes can be prepared from the sequence obtained. These probes can then be used to isolate DNA encoding the complete THP from DNA libraries.

SEQ ID NOs:2 and 5 represent isoforms of naturally occurring *Tenebrio molitor* THP cDNA. They are not complete DNA gene sequences. However, the partial antifreeze nucleic acid sequence of SEQ ID NO:2 or 5 can be completed according to standard methods well known to those of skill in the art. A preferred approach for DNA isolation is RACE. Briefly, this technique involves using PCR to amplify a cDNA sequence using a random 5' primer and a defined 3' primer (5' RACE) or a random 3' primer and a defined 5' primer (3' RACE). The amplified sequence is then subcloned into a vector where it is then sequenced using standard techniques. The RACE method is well known to those of skill in the art and kits to perform RACE are commercially available (e.g. 5' RACE System, GIBCO BRL, Grand Island, New York, USA).

3. Cloning of THP-encoding Inserts into Bacteria

As described above, to prepare and screen genomic DNA total DNA is fragmented and inserted into bacteriophage. Once inserts containing antifreeze nucleic acid sequence of interest have been identified (by PCR, hybridization or the like), they are excised out of λ phage vectors and inserted into bacterial vectors for expansion. Typically, suitable bacterial vectors are known to practitioners in the art and are commercially available. The most suitable vector may depend on the bacteria to be used, the size of the

insert, the method of detection of bacteria which contain the insert of interest and the preference of the practitioner.

To simplify identification of colonies of bacteria transformed with vectors containing the inserts, many vectors have restriction enzyme sites or other splicing sites located within a coding sequence for an enzyme, in particular, β -galactosidase. If an insert has successfully been inserted into the vector at the restriction or splicing sites, the encoded enzyme is inactivated. After transformation of the bacteria with the vector (see, *infra*), colonies grown in the presence of isopropyl β -D-thiogalactoside (IPTG) (a substrate for β -galactosidase) appear white, while the colonies derived from a bacteria which did not incorporate the insert appear blue. Thus, if the frequency of ligation of the insert into the vector was low, one can pick the few colonies that contain inserts over the many that will not.

The vectors are then introduced into variants of *Escherichia coli*. Methods used to introduce foreign DNA into bacterial cells are known to those of skill in the art, but the most frequently used are electroporation and heat shock of competent cells. Most typically, competent *E. coli* are provided commercially (for example from Invitrogen, San Diego, CA). Alternatively, the bacteria can be made competent to take up foreign DNA by techniques well known in the art (see, Sambrook, *et al.*). To introduce the vectors containing the insert of interest into bacteria, the competent bacteria undergo a heat shock process. Briefly, the bacteria are held in an ice water bath and after the DNA has been added, the temperature of the bacteria is raised to 40-50°C, preferably 42°C. The bacteria are returned to the ice bath and then cultured. For a more detailed description of introducing DNA into bacteria see Sambrook, *et al.*, which is incorporated by reference herein.

The bacterial cultures are grown and then plated out on agar. Typically, the vector encodes a gene which confers resistance to an antibiotic to the transformed bacteria. Therefore, bacteria which have taken up the vector survive and form colonies on agar plates permeated with the antibiotic. The surviving colonies can be used to incubate broth and expanded into large cultures of transformed bacteria.

Plasmids and other vectors can be purified from bacterial lysates by methods well known in the art. Many commercial suppliers sell kits for purifying small circularized

DNA (plasmids) from total bacterial DNA. These kits are easy to use and, by following the manufacturer's instructions, the yield is usually quite high.

4. Sequencing of THP DNA

Sequencing of newly isolated DNA will identify and characterize THP nucleic acid of the invention, this nucleic acid encoding THP species or allelic variations, the antifreeze proteins of the invention. A protein can be considered a THP protein isoform if it has at least 60% amino acid sequence identity to the *Tenebrio molitor* THP identified by SEQ ID NO:6 and SEQ ID NO:7.

THP coding sequences can be sequenced while they are still present as inserts in vectors or as inserts released and isolated from the vectors. THP-encoding inserts can be released from the vectors by restriction enzymes or amplified by PCR. For sequencing of the inserts to discover which inserts contain full length antifreeze coding sequences, primers based on the N- or C- terminus, such as those of SEQ ID NO:8 and SEQ ID NO:9, or insertion points in the original λ -phage, can be used as primers. Most preferably, additional primers are synthesized to provide for overlapping sequences. Typically, dideoxy sequencing is done (Sequenase®, U.S. Biochemical). However, other kits and methods are available and known to those of skill in the art.

As an alternative to the bacterial cloning described above, the positive λ -phage plaques from the cDNA library can be subjected to *in vivo* excision with helper phage, for example R408 (Stratagene). The double stranded DNA can then be purified and sequenced or amplified by PCR using primers derived from the λ -phage.

C. Nucleic Acid Hybridization Techniques

The hybridization techniques disclosed herein can be utilized to identify, isolate and characterize genes and gene products (*i.e.*, mRNA) encoding for the antifreeze proteins of the invention, including different THP species and allelic variations.

A variety of methods for specific DNA and RNA measurement using nucleic acid hybridization techniques are known to those of skill in the art. See NUCLEIC ACID HYBRIDIZATION, A PRACTICAL APPROACH, Ed. Hames, B.D. and Higgins, S.J., IRL Press, 1985; Gall & Pardue, *Proc. Natl. Acad. Sci., U.S.A.* **63** 378 (1969), John, et al., *Nature*

223:582 (1969); and Sambrook, et al The selection of a hybridization format is not critical.

For example, one method for evaluating the presence or absence of DNA encoding antifreeze protein in a sample involves a Southern transfer. Briefly, digested genomic DNA is run on agarose slab gels in buffer and transferred to membranes. Hybridization is carried out using nucleic acid probes For the THP of this invention, the nucleic acid probes can be designed based on conserved nucleic acid sequences amongst the class of proteins Preferably nucleic acid probes are 20 bases or longer in length (See Sambrook, *et al.* for methods of selecting nucleic acid probe sequences for use in nucleic acid hybridization.) In the instant invention, preferable probes would be the sequences identified as SEQ ID NO:2 or 5 or the entire coding region of any of the isoforms. Visualization of the hybridized portions allows the qualitative determination of the presence or absence of DNA encoding antifreeze protein.

Similarly, a northern transfer may be used for the detection of mRNA encoding antifreeze protein. In brief, mRNA is isolated from a given cell sample using one of a variety of extraction methods such as an acid guanidinium-phenol-chloroform The mRNA is then electrophoresed to separate the mRNA species and the mRNA is transferred from the gel to a membrane. As with the Southern transfers, labeled probes are used to identify the presence or absence of THP mRNAs.

Sandwich assays are commercially useful hybridization assays for detecting or isolating nucleic acid sequences. Such assays utilize a "capture" nucleic acid covalently immobilized to a solid support and a labeled "signal" nucleic acid in solution The clinical sample will provide the target nucleic acid. The "capture" nucleic acid and "signal" nucleic acid probe hybridize with the target nucleic acid to form a "sandwich" hybridization complex. To be effective, the signal nucleic acid cannot hybridize with the capture nucleic acid.

Typically, labeled signal nucleic acids are used to detect hybridization Complementary nucleic acids or signal nucleic acids may be labeled by any one of several methods typically used to detect the presence of hybridized polynucleotides The most common method of detection is the use of autoradiography or autofluorography with ^3H , ^{125}I , ^{35}S , ^{14}C , or ^{32}P -labeled probes or the like. Other labels include ligands which bind to

labeled antibodies, fluorophores, chemiluminescent agents, enzymes, and antibodies which can serve as specific binding pair members for a labeled ligand. Detection would then depend on the label used.

Detection of a hybridization complex may require the binding of a signal generating complex to a duplex of target and probe polynucleotides or nucleic acids. Typically, such binding occurs through ligand and anti-ligand interactions as between a ligand-conjugated probe and an anti-ligand conjugated with a signal.

The label may also allow indirect detection of the hybridization complex. For example, where the label is a hapten or antigen, the sample can be detected by using antibodies. In these systems, a signal is generated by attaching fluorescent or enzyme molecules to the antibodies or, in some cases, by attachment to a radioactive label (see, Tijssen).

The sensitivity of the hybridization assays may be enhanced through use of a nucleic acid amplification system which multiplies the target nucleic acid being detected. *In vitro* amplification techniques suitable for amplifying sequences for use as molecular probes or for generating nucleic acid fragments for subsequent subcloning are known. Examples of techniques sufficient to direct persons of skill through such *in vitro* amplification methods, including PCR, LCR, Q β -replicase amplification and other RNA polymerase mediated techniques (*e.g.*, NASBATM) are found in Berger, Sambrook, and Ausubel, as well as Mullis *et al.*, U.S. Patent No. 4,683,202; Arnheim & Levinson, *C&EN* 36 (1990); Lomell *et al.*, *J. Clin. Chem.*, 35:1826 (1989); Van Brunt, *Biotechnology* 8:291-294 (1990); Wu & Wallace, *Gene* 4:560 (1989), Sooknanan & Malek, *Biotechnology* 13:563 (1995); Innis; Kwok; Guatelli; Landegren; and Barringer. Improved methods of cloning *in vitro* amplified nucleic acids are described in Wallace *et al.*, U.S. Pat. No. 5,426,039. Other methods recently described in the art are the nucleic acid sequence based amplification (NASBATM, Cangene, Mississauga, Ontario) and Q β -replicase systems. These systems can be used to directly identify mutants where the PCR or LCR primers are designed to be extended or ligated only when a select sequence is present. Alternatively, the select sequences can be generally amplified using, for example, nonspecific PCR primers and the amplified target region later probed for a specific sequence indicative of a mutation.

Oligonucleotides for use as probes, *e.g.*, in *in vitro* amplification methods, as gene probes in diagnostic methods, or as inhibitor components (see below) are typically synthesized chemically according to the solid phase phosphoramidite triester method described by Beaucage and Caruthers, *e.g.*, using an automated synthesizer, as described in Needham-VanDevanter. Purification of oligonucleotides, where necessary, is typically performed by native acrylamide gel electrophoresis or by anion-exchange HPLC as described in Pearson & Regnier. The sequence of the synthetic oligonucleotides can be verified using the chemical degradation method of Maxam & Gilbert.

It will be appreciated that nucleic acid hybridization assays can also be performed in an array-based format. In this approach, arrays bearing a multiplicity of different "probe" nucleic acids are hybridized against a target nucleic acid. In this manner a large number of different hybridization reactions can be run essentially "in parallel". This provides rapid, essentially simultaneous, evaluation of a wide number of reactants. Methods of performing hybridization reactions in array based formats are well known to those of skill in the art (*see, e.g.*, Jackson, *et al.*, *Nature Biotechnology* **14**:1685 (1996), and Chee, *et al.*, *Science* **274**:610 (1995)).

An alternative means for determining the level of expression of a gene encoding a protein is *in situ* hybridization. *In situ* hybridization assays are well known and are generally described in Angerer, *et al.*, *Methods Enzymol.*, **152**:649 (1987). In an *in situ* hybridization assay, cells or tissues are fixed to a solid support, typically a glass slide. If DNA is to be probed, the cells are denatured with heat or alkali. The cells are then contacted with a hybridization solution at a moderate temperature to permit annealing of labeled probes specific to the nucleic acid sequence encoding the protein. The probes are preferably labeled with radioisotopes or fluorescent reporters.

D. Expression of THP

After the coding region of an antifreeze protein gene has been identified, the expression of natural or synthetic antifreeze-encoding nucleic acids can be achieved by operably linking the coding region of an antifreeze protein gene to a promoter (which is either constitutive or inducible), incorporating the construct into an expression vector, and introducing the vector into a suitable host cell. Typical vectors contain transcription and

The nucleic acids (e.g., promoters and vectors) used in the present method can be isolated from natural sources, obtained from such sources as ATCC or GenBank libraries, or prepared by synthetic methods. Synthetic nucleic acids can be prepared by a variety of solution or solid phase methods. Detailed descriptions of the procedures for solid phase synthesis of nucleic acids by phosphite-triester, phosphotriester, and H-phosphonate chemistries are widely available. See, for example, Itakura, U S Pat No 4,401,796; Carruthers, *et al.*, U.S. Pat Nos. 4,458,066 and 4,500,707, Beaucage & Carruthers; Matteucci; Carruthers, *et al.*, *Genetic Engineering* 4:1 (1982), Jones, chapter 2, Atkinson, *et al.*, chapter 3, and Sproat, *et al.*, chapter 4, in OLIGONUCLEOTIDE SYNTHESIS A PRACTICAL APPROACH, Gait (ed.), IRL Press, Washington D.C (1984), Froehler, *et al.*, *Tetrahedron Lett.* 27:469 (1986), Froehler, *et al.*, *Nucleic Acids Res.* 14:5399 (1986); Sinha, *et al.*, *Tetrahedron Lett.* 24:5843 (1983); and Sinha, *et al.*, *Nucl. Acids Res.* 12:4539-4557 (1984), which are incorporated herein by reference

12:4539-4557 (1984), which are incorporated herein by reference

There are several well-known methods of introducing nucleic acids into bacterial cells, any of which may be used in the present invention (see Sambrook, *et al.*). These can include fusion of the recipient cells with bacterial protoplasts containing the DNA, DEAE dextran, infection with viral vectors, and the like

5 The *in vitro* delivery of nucleic acids into bacterial hosts can be to any cell grown in culture. Contact between the cells and the genetically engineered nucleic acid constructs, when carried out *in vitro*, takes place in a biologically compatible medium. The concentration of nucleic acid varies widely depending on the particular application, but is generally between about 1 μ M and about 10 mM. Treatment of the cells with the nucleic acid is generally carried out at physiological temperatures (about 37°C) for periods of time
10 of from about 1 to 48 hours, preferably of from about 2 to 4 hours.

Bacterial strains which can be used to express exogenous nucleic acid include *Escherichia coli*, *Streptococcus cremoris*, *Streptococcus lactis*, *Streptococcus thermophilus*, *Leuconostoc citrovorum*, *Leuconostoc mesenteroides*, *Lactobacillus acidophilus*, *Lactobacillus lactis*, *Bifidobacterium bifidum*, *Bifidobacteriu breve*, and *Bifidobacterium longum*.

In addition to bacterial expression systems, the THP of this invention can be expressed in other systems, in particular yeast and baculovirus, but also in mammalian and plant cells. The system used will depend on the lack of success in other systems, the ability to fold the THP properly, and the eventual use of the THP. For example, if the THP are to be used to protect bread dough yeast from freezing (see, U.S. Patent No. 5,118,792), a yeast system will be used. Yeast strains which can be used to express exogenous nucleic acid include *Torulopsis holmil*, *Saccharomyces fragilis*, *Saccharomyces cerevisiae*, *Saccharomyces lactis*, and *Candida pseudotropicalis*. If plants which can live through
25 freezing temperatures are desired, transgenic techniques can be used to make transgenic plants. In addition, as will be described below, there may be uses for THP in animals, including insects, fish and crustaceans. However, of course, the system used should give THP with comparable thermal hysteresis activity to that found in the *Tenebrio* larvae.

As an example of alternative expression systems, International Publication
30 WO 96/11586 (U.S. Patent Application 08/321,991, filed October 12, 1994), which is incorporated by reference herein, describes the use of fish AFP-transformed *Lactobacillus*

bulgaricus and *Streptococcus thermophilus* to secrete AFP in order to prevent ice recrystallization in fermented frozen foods, in particular frozen yogurt

1. Preparation of Recombinant Vectors

To use isolated sequences in the above techniques, recombinant DNA vectors suitable for transformation of cells are prepared. Techniques for transforming a wide variety of animal and plant cells are well known and described in the technical and scientific literature. See, for example, Weising, *et al.*, *Ann. Rev. Genet.* 22:421 (1988) for plant cells and Sambrook for animal and bacterial cells.

A DNA sequence coding for the desired antifreeze protein, for example a cDNA sequence encoding the full length THP, will preferably be combined with transcriptional and translational initiation regulatory sequences which will direct the transcription of the sequence from the gene in the cells or the intended tissues of the transgenic higher organism. A wide variety of well known transcriptional regulatory elements such as promoters and enhancers can also be included in the vectors selected to express a THP of the invention. Promoters which direct the THP of this invention in their native state can be identified by analyzing the 5' sequences of a genomic clone corresponding to the antifreeze protein genes described herein. Sequences characteristic of promoter sequences can be used to identify the promoter. Sequences controlling eukaryotic gene expression have been extensively studied. For instance, promoter sequence elements include the TATA box consensus sequence (TATAAT), which is usually 20 to 30 base pairs upstream of the transcription start site. In most instances the TATA box is required for accurate transcription initiation.

In construction of recombinant expression cassettes of the invention, a promoter fragment, either related to the THP of this invention or heterologous to the THP, may be employed which will direct expression of the gene in all tissues of a transgenic organism. Such promoters are referred to herein as "constitutive" promoters and are active under most environmental conditions and states of development or cell differentiation. Examples of constitutive promoters of plants include the cauliflower mosaic virus (CaMV) 35S transcription initiation region, the 1'- or 2'- promoter derived from T-DNA of

Agrobacterium tumefaciens, and other transcription initiation regions from various plant genes known to those of skill.

Alternatively, the promoter may direct expression of the polynucleotide of the invention in a specific tissue (tissue-specific promoters) or may be otherwise under more precise environmental control (inducible promoters). Examples of tissue-specific plant promoters under developmental control include promoters that initiate transcription only in certain tissues, such as fruit, seeds, or flowers. The tissue specific E8 promoter from tomato is particularly useful for directing gene expression so that a desired gene product is located in fruits. Other suitable promoters include those from genes encoding embryonic storage proteins. Examples of environmental conditions that may affect transcription by inducible promoters include anaerobic conditions, elevated temperature, or the presence of light.

If proper polypeptide expression is desired, a polyadenylation region at the 3'-end of the coding region should be included. The polyadenylation region can be derived from the natural gene, from a variety of other plant genes, or from T-DNA.

The vector comprising the sequences (*e.g.*, promoters or coding regions) from genes of the invention will typically comprise a marker gene which confers a selectable phenotype on transformed cells. For example, the marker may encode antibiotic resistance, particularly resistance to kanamycin, G418, bleomycin or hygromycin.

Plants can be transformed using viral vectors, such as, for example, the tobacco mosaic virus, to express THP proteins of the invention. Selection and construction of vectors and techniques for transforming a wide variety of plant cells are well known, for example, see Hamamoto, et al., U.S. Patent No. 5,618,699.

2. Production of Transgenic Organisms

DNA constructs of the invention may be introduced into the genome of a host by a variety of conventional techniques. For example, in plants, the DNA construct may be introduced directly into the genomic DNA of the plant cell using techniques such as electroporation and microinjection of plant cell protoplasts, or the DNA constructs can be introduced directly to plant tissue using ballistic methods, such as DNA particle bombardment. As discussed above, plant virus vectors such as tobacco mosaic virus

containing the THP sequences of the invention can be used to inoculate a plant.

Alternatively, the DNA constructs may be combined with suitable T-DNA flanking regions and introduced into a conventional *Agrobacterium tumefaciens* host vector. The virulence functions of the *Agrobacterium tumefaciens* host will direct the insertion of the construct and adjacent marker into the plant cell DNA when the cell is infected by the bacteria.

Agrobacterium tumefaciens-mediated transformation techniques, including disarming and use of binary vectors, are well described in the scientific literature. See, for example Horsch, *et al.*, *Science* **233**:496 (1984), and Fraley, *et al.*, *Proc. Nat'l. Acad. Sci. USA* **80**:4803 (1983).

Transformed plant cells which are derived by any of the above transformation techniques can be cultured to regenerate a whole plant which possesses the transformed genotype and thus the desired phenotype, such as increased tolerance to freezing. The transformed plants of the invention can also be employed as "living factories" to express an antifreeze protein in substantial quantities. Such plant regeneration techniques rely on manipulation of certain phytohormones in a tissue culture growth medium, typically relying on a biocide and/or herbicide marker which has been introduced together with the desired nucleotide sequences. Plant regeneration from cultured protoplasts is described in Evans, *et al.*, PROTOPLASTS ISOLATION AND CULTURE, HANDBOOK OF PLANT CELL CULTURE, pp 124-176, Macmillan Publishing Company, New York, 1983; and Binding, *REGENERATION OF PLANTS, PLANT PROTOPLASTS*, pp 21-73, CRC Press, Boca Raton, 1985. Regeneration can also be obtained from plant callus, explants, organs, or parts thereof. Such regeneration techniques are described generally in Klee, *et al.*, *Ann. Rev. of Plant Phys.* **38**:467 (1987).

To produce a transgenic plant or animal, for example a salt-water fish, microinjection techniques are known in the art and well described in the scientific and patent literature. The introduction of DNA constructs into cells using polyethylene glycol precipitation is described in Paszkowski, *et al.*, *EMBO J.* **3**:2717 (1984). Electroporation techniques are described in Fromm, *et al.*, *Proc. Natl. Acad. Sci. USA* **82**:5824 (1985). Ballistic transformation techniques are described in Klein, *et al.*, *Nature* **327**:70 (1987).

III. DETECTION AND CHARACTERIZATION OF THE CLASS OF THP OF THIS INVENTION

By the assays described below, the THP of this invention share characteristics with a thermal hysteresis protein isolated from final larval instar *Tenebrio molitor* hemolymph. These assays are used to define whether other novel THP are sufficiently related to the prototype proteins YL-1 through YL-4 so as to fall within the scope of this invention. The assays can also be used to detect and quantify THPs present in bacteria broth, tissue culture fluid and plant and animal tissues.

A. Detection of THP

Expressed THP may be detected or quantified by a variety of methods. Preferred methods involve the use of functional activity assays and immunological assays utilizing specific antibodies

1. Antibodies

Methods of producing polyclonal and monoclonal antibodies are known to those of skill in the art. See, e.g., Coligan, CURRENT PROTOCOLS IN IMMUNOLOGY, Wiley/Greene, NY (1991); Stites *et al.* (eds.) BASIC AND CLINICAL IMMUNOLOGY (7th ed.) Lange Medical Publications, Los Altos, CA, and references cited therein ("Stites"); Goding, MONOCLONAL ANTIBODIES. PRINCIPLES AND PRACTICE (2d ed.) Academic Press, New York, NY (1986), Kohler & Milstein, *Nature* 256:495 (1975); and Harlow and Lane. Such techniques include antibody preparation by selection of antibodies from libraries of recombinant antibodies in phage or similar vectors. See, Huse *et al.*, *Science* 246:1275 (1989) ("Huse"); and Ward *et al.*, *Nature* 341:544 (1989)

To produce large amounts of antibodies for use in, for example, immunoaffinity purification, a number of immunogens may be used THP from *Tenebrio molitor* or from the transformed cells as described in this invention are the preferred immunogens for the production of monoclonal or polyclonal antibodies. Naturally occurring antifreeze protein from other organisms may also be used either in pure or impure form. Synthetic peptides made using a fragment of antifreeze protein sequence described

herein may also used as an immunogen for the production of antibodies to the protein. The peptides can be used alone or conjugated to another composition.

Methods of production of polyclonal antibodies are known to those of skill in the art. In brief, an immunogen is mixed with an adjuvant, as described above, and animals are immunized. The animal's immune response to the immunogen preparation is monitored by taking test bleeds and determining the titer of reactivity to the immunogen. When appropriately high titers of antibody to the immunogen are obtained, blood is collected from the animal and antisera are prepared. Further fractionation of the antisera to enrich for antibodies reactive to the protein can be done if desired. (*See Harlow and Lane, supra*).

Large amounts of monoclonal antibodies for use in immunoaffinity purification or immunoassays may be obtained by various techniques familiar to those skilled in the art. Briefly, spleen cells from an animal immunized with a desired antifreeze protein are immortalized, commonly by fusion with a myeloma cell (*See, Kohler & Milstein, Eur. J. Immunol. 6:511 (1976), incorporated herein by reference*). Alternative methods of immortalization include transformation with Epstein Barr Virus, oncogenes, or retroviruses, or other methods well known in the art. Colonies arising from single immortalized cells are screened for production of antibodies of the desired specificity and affinity for THP. The yield of the monoclonal antibodies produced by such cells may be enhanced by various techniques, including injection into the peritoneal cavity of a vertebrate host. Alternatively, one may isolate DNA sequences which encode a monoclonal antibody or a binding fragment thereof by screening a DNA library from human B cells according to the general protocol outlined in Huse

The concentration of THP can be measured by a variety of immunoassay methods. For a review of immunological and immunoassay procedures in general, see Stites. Moreover, the immunoassays of the present invention can be performed in any of several configurations, which are reviewed extensively in *ENZYME IMMUNOASSAY*, E.T. Maggio, ed., CRC Press, Boca Raton, Florida (1980), Tijssen, and Harlow and Lane, each of which is incorporated herein by reference.

For example, in order to produce antisera for use in an immunoassay for antifreeze protein, YL-1, YL-2, YL-3, YL-4 or fragments thereof, are isolated as described

herein. An inbred strain of mice or rabbits is immunized with the above antifreeze isoforms or polypeptide of SEQ ID NO 4 using a standard adjuvant, such as Freund's adjuvant, and a standard immunization protocol. Alternatively, a synthetic peptide derived from the sequences disclosed herein and conjugated to a carrier protein can be used as an immunogen.

5 Polyclonal sera are collected and titered against the THP in an immunoassay, for example, a solid phase immunoassay with the THP immobilized on a solid support. Polyclonal antisera with a K_a of 10^4 M^{-1} or greater are selected and tested for their cross reactivity against homologous proteins from other organisms and/or non-antifreeze protein, using a competitive binding immunoassay. Specific monoclonal and polyclonal antibodies and
10 antisera will usually bind with a K_D of at least about 0.1 mM, more usually at least about 1 μM , preferably at least about 0.1 μM or better, and most preferably, 0.01 μM or better.

2. Immunological Binding Assays.

In a preferred embodiment, THP are detected and/or quantified using any of
5 a number of well recognized immunological binding assays (*see, e.g.*, U.S. Patents 4,366,241; 4,376,110; 4,517,288; and 4,837,168). For a review of the general immunoassays, see also METHODS IN CELL BIOLOGY Vol. 37: *Antibodies in Cell Biology*, Asai, ed. Academic Press, Inc. New York (1993), and Stites. Immunological binding assays (or immunoassays) typically utilize a "capture agent" to specifically bind to and
10 often immobilize the analyte (in this case THP or a fragment thereof). The capture agent is a moiety that specifically binds to the analyte. In a preferred embodiment, the capture agent is an antibody that specifically binds to THP. The antibody (anti-THP) may be produced by any of a number of means well known to those of skill in the art and as described above.

Immunoassays also often utilize a labeling agent to specifically bind to and
25 label the binding complex formed by the capture agent and the analyte. The labeling agent may itself be one of the moieties comprising the antibody/analyte complex. Thus, the labeling agent may be a labeled THP polypeptide or a labeled anti-THP antibody. Alternatively, the labeling agent may be a third moiety, such as another antibody, that specifically binds to the antibody/THP complex.

30 In a preferred embodiment, the labeling agent is a second THP antibody bearing a label. Alternatively, the second THP antibody may lack a label, but it may, in

turn, be bound by a labeled third antibody specific to antibodies of the species from which the second antibody is derived. The second can be modified with a detectable moiety, such as biotin, to which a third labeled molecule can specifically bind, such as enzyme-labeled streptavidin

5 Other proteins capable of specifically binding immunoglobulin constant regions, such as protein A or protein G may also be used as the label agent. These proteins are normal constituents of the cell walls of streptococcal bacteria. They exhibit a strong non-immunogenic reactivity with immunoglobulin constant regions from a variety of species (see, generally Kronval, *et al.*, *J. Immunol.* **111**:1401-1406 (1973), and Akerstrom, *et al.*,
10 *J. Immunol.* **135**:2589-2542 (1985)).

Throughout the assays, incubation and/or washing steps may be required after each combination of reagents. Incubation steps can vary from about 5 seconds to several hours, preferably from about 5 minutes to about 24 hours. However, the incubation time will depend upon the assay format, analyte, volume of solution, concentrations, and the like. Usually, the assays will be carried out at ambient temperature, although they can
5 be conducted over a range of temperatures, such as 4°C to 40°C

a. Non-Competitive Assay Formats.

Immunoassays for detecting THP may be either competitive or
10 noncompetitive. Noncompetitive immunoassays are assays in which the amount of captured analyte (in this case THP) is directly measured. In one preferred "sandwich" assay, for example, the capture agent (anti-THP antibodies) can be bound directly to a solid substrate where they are immobilized. These immobilized antibodies then capture protein present in the test sample. The THP thus immobilized is then bound by a labeling agent,
25 such as a second THP antibody bearing a label. Alternatively, the second THP antibody may lack a label, but it may, in turn, be bound by a labeled third antibody specific to antibodies of the species from which the second antibody is derived. The second can be modified with a detectable moiety, such as biotin, to which a third labeled molecule can specifically bind, such as enzyme-labeled streptavidin.

b Competitive Assay Formats.

In competitive assays, the amount of analyte (THP) present in the sample is measured indirectly by measuring the amount of an added (exogenous) analyte (THP) displaced (or competed away) from a capture agent (anti THP antibody) by the analyte present in the sample. In one competitive assay, a known amount of, in this case THP, is added to the sample and the sample is then contacted with a capture agent, in this case an antibody that specifically binds THP. The amount of THP bound to the antibody is inversely proportional to the concentration of THP present in the sample.

In a particularly preferred embodiment, the antibody is immobilized on a solid substrate. The amount of THP bound to the antibody may be determined either by measuring the amount of THP present in an THP/antibody complex, or alternatively by measuring the amount of remaining uncomplexed THP. The amount of THP may be detected by providing a labeled THP molecule.

A hapten inhibition assay is another preferred competitive assay. In this assay a known analyte, in this case THP, is immobilized on a solid substrate. A known amount of anti-THP antibody is added to the sample, and the sample is then contacted with the immobilized THP. In this case, the amount of anti-THP antibody bound to the immobilized THP is inversely proportional to the amount of THP present in the sample. Again the amount of immobilized antibody may be detected by detecting either the immobilized fraction of antibody or the fraction of the antibody that remains in solution. Detection may be direct where the antibody is labeled or indirect by the subsequent addition of a labeled moiety that specifically binds to the antibody as described above.

Immunoassays in the competitive binding format can be used for crossreactivity determinations to permit one of skill to determine if a novel THP is sufficiently related to the claimed THP so as to fall under the claims of this invention. For example, a THP fragment of SEQ ID NO:4 can be immobilized to a solid support. Proteins are added to the assay which compete with the binding of the antisera to the immobilized antigen. The ability of the proteins to compete with the binding of the antisera to the immobilized THP is compared to the binding by the same THP as was used to coat the solid support. The percent crossreactivity for the above proteins is calculated, using standard calculations. Those antisera with less than 10% crossreactivity with the THP of SEQ ID

NO:4 are selected and pooled. The cross-reacting antibodies are optionally removed from the pooled antisera by immunoabsorption with the above proteins

The immunoabsorbed and pooled antisera can be used in a competitive binding immunoassay, as described above, to analyze whether a second protein is an antifreeze protein of this invention. In the competitive binding immunoassay the protein, or immunogen, used to develop the antiserum competes with a second, uncharacterized protein or peptide in an antibody binding reaction. The two proteins are each assayed at a wide range of concentrations and the amount of each protein required to inhibit 50% of the binding of the antisera to the immobilized protein is determined. If the amount of the second protein required is less than 10 times the amount of the characterized immunogen (for example, SEQ ID NO.2, SEQ ID NO.4 or an immunogenic fragment thereof) that is required, then the second protein is said to specifically bind to an antibody generated to that characterized (antifreeze protein) immunogen.

c. Other Assay Formats.

Western blot (immunoblot) analysis can be used to detect and quantify the presence of antifreeze protein in a sample. The technique generally comprises separating sample proteins by gel electrophoresis on the basis of molecular weight, transferring the separated proteins to a suitable solid support, (such as a nitrocellulose filter, a nylon filter, or derivatized nylon filter), and incubating the sample with the antibodies that specifically bind THP. The anti-THP antibodies specifically bind to THP on the solid support. These antibodies may be directly labeled or alternatively may be subsequently detected using labeled antibodies (*e.g.*, labeled sheep anti-mouse antibodies) that specifically bind to the anti-antifreeze protein.

In addition to using nucleic acid probes for identifying novel forms of the class of proteins claimed herein, it is possible to use antibodies to probe expression libraries. This is a well known technology (See Young & Davis, *Proc. Nat'l Acad. Sci. USA* **80**:1194 (1982)). In general, a cDNA expression library may be prepared from commercially available kits or using readily available components. Phage vectors are preferred, but a variety of other vectors are available for the expression of protein. Such vectors include but are not limited to yeast, animal cells and *Xenopus* oocytes. One selects mRNA from a

source that is enriched with the target protein and creates cDNA which is then ligated into a vector and transformed into the library host cells for immunoscreening. Screening involves binding and visualization of antibodies bound to specific proteins on cells or immobilized on a solid support such as nitrocellulose or nylon membranes. Positive clones are selected for purification to homogeneity and the isolated cDNA then prepared for expression in the desired host cells. A good general review of this technology can be found in METHODS OF CELL BIOLOGY, VOL. 37 entitled *Antibodies in Cell Biology*, Assai (ed.) 1993.

Where the antibodies are generated to protein like the THPs of this invention, which are rich in cysteine and hypothetically contain many disulfide bridges, the test proteins are optionally denatured to fully test for selective binding and it may be best to measure the test proteins against proteins of similar size, *e.g.*, one would test a full length THP against a prototype full length THP even though the antisera was generated against a fragment of the prototype THP. This simplifies the test and avoids having to take into account conformational problems and molecular weight/molar concentrations in the determination of the results from the competitive immunoassays

Other assay formats include liposome immunoassays (LIA), which use liposomes designed to bind specific molecules (*e.g.*, antibodies) and release encapsulated reagents or markers. The released chemicals are then detected according to standard techniques (*see*, Monroe *et al.*, *Amer. Clin. Prod. Rev.* 5:34 (1986)).

B. Purification of THP

The polypeptides of this invention may be purified to substantial purity by standard techniques, from a variety of sources such as larval hemolymph, tissue culture media, transgenic plants and animals, yeasts and bacteria. For standard purification procedures, including selective precipitation with such substances as ammonium sulfate; column chromatography, immunopurification methods, and others see, for instance, Scopes, *PROTEIN PURIFICATION PRINCIPLES AND PRACTICE*, Springer-Verlag, New York (1982), U.S. Patent No. 4,673,641, Ausubel, and Sambrook, all incorporated herein by reference.

1. Purification of THP from Bacterial Cultures

In the case of secreted proteins, the protein of interest can be isolated and purified from the broth in which bacteria have been grown without having to resort to the cell lysis methods detailed below

2. Purification of THP from Bacterial Cytoplasm and Periplasm

After expression of THP in *E. coli*, the protein may be found in the periplasm, cytoplasm or inclusion bodies of the bacteria. The periplasmic fraction of the bacteria can be isolated by cold osmotic shock in addition to other methods known to those of skill in the art (see Ausubel, and Trayer, & Buckley, *J. Biol. Chem.* 245(18):4842 (1970)).

To isolate proteins from the periplasm and cytoplasm, the bacterial cells are centrifuged to form a pellet. The pellet is resuspended in a buffer containing 20% sucrose. To lyse the cells, the bacteria are centrifuged and the pellet is resuspended in ice-cold 5 mM $MgSO_4$ and kept in an ice bath for approximately 10 minutes. The cell suspension is centrifuged and the supernatant decanted and saved. Alternatively, the bacteria can be lysed by sonication. The proteins present in the supernatant can be separated from the host proteins by standard separation techniques well known to those of skill in the art

3. Purification of Inclusion Bodies

When recombinant proteins are expressed by the transformed bacteria in large amounts, typically after promoter induction; but expression can be constitutive, the proteins may form insoluble aggregates.

Purification of aggregate proteins (hereinafter referred to as inclusion bodies) involves the extraction, separation and/or purification of inclusion bodies by disruption of bacterial cells, typically but not limited by, incubation in a buffer of about 100-150 $\mu g/mL$ lysozyme and 0.1% NONIDET P40®, a non-ionic detergent. The cell suspension can be ground using a Polytron grinder (Brinkman Instruments, Westbury, N.Y.). Alternatively, the cells can be sonicated on ice. Alternate methods of lysing bacteria are described in Ausubel and Sambrook and will be apparent to those of skill in the art.

The cell suspension is centrifuged and the pellet containing the inclusion bodies resuspended in buffer, *e.g.*, 20 mM Tris-HCl (pH 7.2), 1 mM EDTA, 150 mM NaCl and 2% TRITON-X 100®, a non-ionic detergent. It may be necessary to repeat the wash step to remove as much cellular debris as possible. The remaining pellet of inclusion bodies may be resuspended in an appropriate buffer (*e.g.* 20 mM sodium phosphate, pH 6.8, 150 mM NaCl). Other appropriate buffers will be apparent to those of skill in the art.

Following the washing step, the inclusion bodies are solubilized by the addition of a solvent that is both a strong hydrogen acceptor and a strong hydrogen donor (or a combination of solvents each having one of these properties) together with a reducing agent such as DTT. The proteins that formed the inclusion bodies can then be renatured by dilution or dialysis with a compatible buffer. Suitable solvents include, but are not limited to urea (from about 4 M to about 8 M), formamide (at least about 80%, volume/volume basis), and guanidine hydrochloride (from about 4 M to about 8 M). Some solvents which are capable of solubilizing aggregate-forming proteins, for example SDS (sodium dodecyl sulfate), 70% formic acid, are inappropriate for use in this procedure due to the possibility of irreversible denaturation of the proteins, accompanied by a lack of immunogenicity and/or activity. Although guanidine hydrochloride and similar agents are denaturants, this denaturation is not irreversible and renaturation may occur upon removal (by dialysis, for example) or dilution of the denaturant, allowing re-formation of immunologically and/or biologically active protein.

After solubilization, the protein can be separated from other bacterial proteins by standard separation techniques.

4. Standard Protein Separation Techniques

a. Solubility Fractionation

Often as an initial step and if the protein mixture is complex, an initial salt or organic solvent fractionation can separate many of the unwanted host cell proteins (or proteins derived from the cell culture media) from the recombinant protein of interest. The preferred salt is ammonium sulfate. Ammonium sulfate precipitates proteins by effectively reducing the amount of water in the protein mixture. Proteins then precipitate on the basis of their solubility. The more hydrophobic a protein is, the more likely it is to precipitate at

lower ammonium sulfate concentrations. A typical protocol is to add saturated ammonium sulfate to a protein solution so that the resultant ammonium sulfate concentration is between 20-30%. This will precipitate the most hydrophobic of proteins. The precipitate is discarded (unless the protein of interest is hydrophobic) and ammonium sulfate is added to the supernatant to a concentration known to precipitate the protein of interest. The precipitate is then solubilized in buffer and the excess salt removed if necessary, either through dialysis or diafiltration. Other methods that rely on solubility of proteins, such as cold ethanol precipitation, are well known to those of skill in the art and can be used to fractionate complex protein mixtures.

b. Size Differential Filtration

If the size of the protein of interest is known or can be estimated from the cDNA sequence, proteins of greater and lesser size can be removed by ultrafiltration through membranes of different pore size (for example, Amicon or Millipore membranes). As a first step, the protein mixture is ultrafiltered through a membrane with a pore size that has a lower molecular weight cut-off than the molecular weight of the protein of interest. The retentate of the ultrafiltration is then ultrafiltered against a membrane with a molecular cut off greater than the molecular weight of the protein of interest. The recombinant protein will pass through the membrane into the filtrate. The filtrate can then be chromatographed as described below.

c. Column Chromatography

Proteins can be separated on the basis of their size, net surface charge, hydrophobicity and affinity for ligands. In addition, antibodies raised against proteins can be conjugated to column matrices and the proteins immunopurified. All of these methods are well known in the art. See Scopes, R. K., Protein Purification: Principles and Practice, 2nd ed., Springer Verlag, (1987).

It will be apparent to one of skill that chromatographic techniques can be performed at any scale and using equipment from many different manufacturers (*e.g.*, Pharmacia Biotech).

In a preferred embodiment, the purification of THP from *E. coli* supernatant is accomplished in part by gel filtration, and protein concentrations are determined according to a number of techniques. *e.g.*, Bradford, *Anal. Biochem.* 72:248-257 (1976)

d. Amino Acid Sequence

The amino acid sequences of the THP of this invention can be determined by, for example, Edman degradation, a technique which is well known in the art. By Edman degradation, an internal section of THP was sequenced spanning a repeating motif of 12 contiguous amino acids which was found to make up the bulk of the THP. This motif (SEQ ID NO:1) is repeated at least 5 times and more preferably 5 to 12 times in each isoform.

In addition to the internal sequencing, N-terminal sequencing can be performed by techniques known in the art. However, in the native THP26 and by analogy in 3 of the recombinant THP isoforms, the N terminus was blocked. By nucleic acid sequencing, the N termini were determined to be a glutamine which would be consistent with N-terminal sequencing being blocked by pyroglutamate (cyclized glutamine). The deduced N-termini of the class of THP of this invention is identified as SEQ ID NO:4.

e. Molecular Weight/Isoelectric Point

The molecular weight of a protein can be determined by many different methods, all known to one of skill in the art. Some methods of determination include: SDS gel electrophoresis, native gel electrophoresis, molecular exclusion chromatography, zonal centrifugation, mass spectroscopy, and calculation from sequencing. Disparity between results of different techniques could be due to factors inherent in the technique. For example, native gel electrophoresis, molecular exclusion chromatography and zonal centrifugation depend factors such as the size, shape and net charge of the protein. The proteins of this invention are rich in cysteine and might be expected to form many disulfide bonds, both intra- and intermolecular. SDS gel electrophoresis depends on the binding of SDS to amino acids present in the protein. Some amino acids bind SDS more tightly than others, therefore, proteins will migrate differently depending on their amino acid composition. In the instant invention, because the THP disclosed herein are repeating units

of a motif, the relative number of different amino acids is quite small and could result in a large disparity in molecular weight by SDS gel electrophoresis. The calculated molecular weight from the sequence utilizes the frequency of amino acids present in the protein and multiplies that frequency by the molecular weight of the amino acid. If the protein is glycosylated, calculated molecular weight will not reflect this and mass spectroscopy may be a necessary alternative

In the present invention, the apparent molecular weight of the class of THP by molecular exclusion chromatography was between 17 and 19 kD; by SDS gel electrophoresis, 22-30 kD. By mass spectroscopy, the molecular weight of the class of proteins was found to be between 7 to 12, preferably 8 to 11 and most preferably from about 8.4 to 11.7 kD. This agreed with the results obtained when the molecular weight was calculated from the amino acid sequencing. By comparing the amino acid sequences and the molecular weights by mass spectroscopy, it is likely that the differences in molecular weight are due largely to different number of motif repeats.

The isoelectric point of a protein can be determined by native gel (or disc) electrophoresis, isoelectric focussing or in a preferred method, by calculation given the amino acid content of the protein. The class of proteins of the instant invention have a calculated pI of about 8 to 10.

f. Functional Assays

The THP species and isoforms of the invention can be identified and characterized by at least two functional properties, for example, thermal hysteresis and unique formation of ice crystals:

(1) Thermal Hysteresis

The proteins of this invention are approximately 100 fold more active than known fish antifreeze proteins in a thermal hysteresis assay. Thermal hysteresis is defined as the difference between the solution freezing and melting temperatures. Freezing point is taken as the temperature at which uncontrollable ice growth occurs from a seed ice crystal. Melting point is taken as the warmest temperature at which an ice crystal can be stably held without melting. TH activity can be measured in a nanoliter osmometer (Clifton Technical Physics, Martford, NY) by methods well known in the art (see, for example. Chakrabartty

& Hew, *Eur. J. Biochem.* **202**:1057 (1991)) The starting ice crystal is usually 20-50 μm in diameter. The buffer used is typically 100 mM NH_4HCO_3 (pH 7.9) but other buffers of similar osmolarity can be used. Alternatively, TH activity can be measured in bacterial broth, tissue culture fluid, or hemolymph.

5 The osmometer is a thermal electric cooling module with a separate but linked variable temperature control. This apparatus allow temperature regulation in the 0°C to -9°C range with a deep freeze mode to -40°C . The cooling module can be set up on a microscope stage where the growth and melt behavior of an ice crystal can be observed directly. The sample holder can be a small plate with dimensions of about 7 mm x 7 mm x
10 0.75 mm containing multiple small sample holes (about 0.35 mm in diameter). A drop of immersion oil, such as Cargille's B immersion oil, can be placed on the underside of the sample holder so that the sample holes are filled. 1 to 5 nL samples are then delivered into the center of the oil-filled hole by a capillary tube.

5 To measure TH activity, the samples are first frozen by cooling the samples rapidly to -40°C and then allowing them to warm up to the melting point temperature. Once the melting point is reached, the samples are cooled by approximately 0.02°C (10 milli-osmoles or mosmoles) per 10-15 seconds until the freezing temperature is reached. The conversion from the unit "osmos" to " $^\circ\text{C}$ " is 1.00 osmoles equals 1.86°C . In most
10 instances, when the freezing point of a THP sample is reached, the ice crystal within the sample will grow spontaneously and rapidly. This leads to freezing of the entire sample.

Alternatively, a small ice crystal can be frozen onto the surface of a solution and the temperature of the solution immediately reduced to below freezing. The temperature below freezing when the nucleated ice crystal begins to grow is the freezing point depression or the thermal hysteresis measurement (see, Patterson & Duman, *J. Exp.*
25 *Zool.* **210**:361 (1979); and Wu, *et al.*, *J. Comp. Physiol. B* **161**:271 (1991). The thermal hysteresis activity of a solution is dependent on the concentration of the antifreeze protein, with the greater the concentration of the protein, the greater the activity shown by the solution. However, increased concentrations of THPs produce incrementally smaller increases in TH activity and a maximum is approached. In other words, the relationship
30 between THP concentration and TH is hyperbolic, not linear.

The proteins of the present invention preferably have thermal hysteresis values greater than 1.0°C at about 1 mg/mL, more preferably greater than 1.5°C at about 1 mg/mL and most preferably between about 1.5-3.0°C at about 1 mg/mL.

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(2) Unique Formation of Ice Crystals

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In addition to having approximately 100 fold more specific activity than previously known fish antifreeze proteins, the proteins of this invention produce different shaped ice crystals. Under microscopic analysis, fish antifreeze proteins produce ice crystals which are hexagonal bipyramids with flat, well-defined facets. The THP of this invention, on the other hand, form crystals with rounded edges, preferably oval-shaped and with non-flat surfaces, preferably convex.

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IV. USE OF ANTIFREEZE PROTEINS AND RELATED GENES

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The proteins or genes encoding the THP may be used in ways to suppress ice crystal growth. For a comprehensive review of uses of antifreeze proteins, see U.S. Patent No. 5,118,792. The THP of this invention may be introduced in the protein form, or they may be introduced as genes which are expressed endogenously at a level which should be attainable by expressing an antifreeze protein in a cell under the control of a suitable strong promoter to produce the proteins. Suitable concentrations of THP will vary depending on the use, but will typically be in the range of from about one part per billion to about one part per thousand (i.e., 1 µg/L to 1 g/L).

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In one embodiment of the invention, the proteins will be introduced to foodstuffs. This has a number of different aspects. One is the introduction into plant foodstuffs, either into the entire plant and thus conferring some degree of general resistance to damage from subfreezing climatic conditions, or into a plant part such as the fruit or vegetable portion to minimize damage specifically to those particular plant organs upon freezing. Exemplary plant parts are stems, roots, leaves, flowers, petioles, pericarp, seeds, vegetative tissue, tubers and so forth.

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The texture, taste, and useful storage life of frozen vegetables will be improved, for example, celery, potatoes, asparagus, peas, carrots, beans, broccoli, sweet

corn and spinach. Similarly, the texture, taste and useful storage life of fruits will be enhanced, including strawberries, blueberries, raspberries, citrus fruits, bananas, grapes, kiwis, peaches, pineapples, plums, cherries, tomatoes and mangoes.

This introduction into plant and other products may be most easily accomplished by genetic introduction of appropriate nucleic acids into the target organism. Expression of the nucleic acid, either constitutively or inducibly, before food processing has begun, or after harvesting and processing has begun, may lead to sufficiently high levels of the polypeptide to effectively protect the foodstuff, such as up to 0.5%, but more preferably up to about 0.1% of total plant protein by mass. Expression can also be on a tissue specific basis. For example, linkage to ripening genes in fruits may result in expression even after harvesting from the producing plant.

The polypeptides may also be added into foods which are expected to be frozen. Many frozen foods are intended to be consumed in the cold state, for example, ice cream, frozen yogurt, ice milk, sherbet, popsicles, frozen whipped cream, frozen cream pies, frozen puddings and the like. In particular, texture and flavor are adversely affected by the formation of large ice crystals throughout a freeze-thaw cycle that occurs in most home frost-free freezers or upon sustained storage in the frozen state. This ice crystal growth process may be prevented entirely, or at least minimized by the addition of antifreeze polypeptides. The purified antifreeze protein may be either incorporated throughout the foodstuff, or may, alternatively, be applied to the surface where condensation and crystal formation is expected to occur most readily.

In another embodiment, the genes that encode the THP of this invention are used to transform microorganisms which when added to foodstuffs, protect the foodstuffs or the microorganism from freezing. For example, bacteria such as *Streptococcus thermophilus* and *Lactobacillus bulgaricus* can be added to dairy products that is intended to be sold as frozen yogurt. In addition to fermenting the dairy products to produce yogurt, the THP expressed by the bacteria will protect the product from home freezer freeze-thaw cycles and produce a more palatable product.

Another use would be to transform dough yeast with nucleic acids encoding THP. Upon incorporation and expression of this gene into the yeast, and use of these yeast in frozen dough, the dough will naturally leaven upon thawing because the yeast viability

will remain high upon thawing. Because less damage accumulates from storage in the presence of these antifreeze polypeptides and thawed samples preserve high viability, either longer storage times will be possible, or perhaps much smaller aliquots will need to be stored.

5 There are various embodiments not specific to the food freezer. One is the use of THP to protect plants from climatic freezing conditions. The THP may be either internally incorporated into the cytoplasm by expression of an introduced gene, or the proteins may be externally applied to the plants. External application may be achieved either by direct application of the proteins to the plant, or by the external deposit onto the plant of
10 an organism which secretes the proteins. These same alternatives for introduction apply to other uses as well.

 In addition to plants, it is envisioned that the THP of this invention can be used to produce transgenic animals, including fish that can withstand sub-zero temperatures. While some polar fish do synthesize antifreeze proteins, most fish do not live
5 in environments where the water temperature drops below 0°C. However, transgenic fish containing exogenous nucleic acid encoding THP could be held and perhaps partially raised in sub-zero salt-water. In particular, salt-water fish being raised in farms, most particularly salmon. In addition to salmon, farmed transgenic prawns are a potential recipient of THP genes.

 In addition to the above embodiments, it is envisioned that the THP of this invention can be used to regulate the expression of endogenous THP genes within a cold-tolerant organism. The expression of antifreeze protein gene products may be increased as a method of preparing, *e.g.*, for subsequent isolation, endogenous antifreeze proteins. Conversely, by downregulating endogenous antifreeze gene expression, an otherwise cold-
25 tolerant pest may be converted to a less aggravating phenotype.

 Methods of altering the expression of endogenous genes are well known to those of skill in the art. Typically such methods involve altering or replacing all or a portion of the regulatory sequences controlling expression of the particular gene that is to be regulated. In a preferred embodiment, the regulatory sequences (*e.g.*, the native promoter)
30 upstream of one or more of the THP are altered.

This is typically accomplished by the use of homologous recombination to introduce a heterologous nucleic acid into the native regulatory sequences. To downregulate expression of one or more THP gene products, simple mutations that either alter the reading frame or disrupt the promoter are suitable. To upregulate expression of the THP gene products, the native promoter(s) can be substituted with heterologous promoter(s) that induce higher than normal levels of transcription.

In a particularly preferred embodiment, nucleic acid sequences comprising the structural gene in question or upstream sequences are utilized for targeting heterologous recombination constructs. Utilizing the structural gene sequence information provided in SEQ ID NOs:2 and 5 or the upstream or downstream sequence information provided in SEQ ID Nos:10, 12, 13 and 14, one of skill in the art can create homologous recombination constructs with only routine experimentation.

The use of homologous recombination to alter expression of endogenous genes is described in detail in U.S. Patent 5,272,071, WO 91/09955, WO 93/09222, WO 96/29411, WO 95/31560, and WO 91/12650. Homologous recombination in mycobacteria is described by Azad, *et al.*, *Proc. Nat'l Acad. Sci. USA* **93**:4787 (1996), Baulard, *et al.*, *J. Bacteriol.* **178**:3091 (1996), and Pelicic, *et al.*, *Mol. Microbiol.* **20**:919 (1996). Homologous recombination in animals has been described by Moynahan, *et al.*, in *Hum. Mol. Genet.* **5**(7):875 (1996) and in plants by Offringa, *et al.*, *EMBO J.* **9**(10):3077 (1990).

Another embodiment is the introduction of an antifreeze protein into aqueous liquids surrounding an organ, tissue or other biological sample. One particular use would be during transportation to a hospital for a transplantation operation or for storage purposes. The antifreeze protein should allow short- or long-term storage at a subfreezing temperature, thereby minimizing inherent metabolism or degradation, but with substantially diminished cellular damage from ice crystal growth. Other medically important temperature sensitive biological samples are blood and blood products, therapeutic agents, protein drugs, bioassay reagents and vaccines.

Yet another embodiment is the introduction of an antifreeze protein into cells or their extracts destined for frozen storage. For example, bacterial cells, yeast cells, plant cells and, most particularly, animal cells containing the THP have increased cell or tissue viability with minimal or no loss of inherent characteristics due to the freeze-thaw

process. Subcellular samples or cellular extracts may have similar sensitivities to freezing, especially on prolonged storage. Typical examples will be *in vitro* protein translation systems, enzyme preparations, and particularly samples which contain sensitive membrane components, such as chloroplast or mitochondrial membrane preparations. In particular, samples containing organelles may display increased resistance to freezing damage upon addition of these antifreeze polypeptides. Soft animal tissues will exhibit less damage upon freezing in the presence of the subject polypeptides, and addition of the polypeptides will be useful in situations when cellular integrity upon freezing and subsequent thawing is important or desired, such as for tissue culture deposits. Thus, samples destined for frozen storage, such as for cell or tissue depositories, might routinely have the proteins added to them. Among the cell types often stored are genetic variants of bacteria, fungi (including yeast), and, particularly, higher eukaryote cells (such as hybridoma strains and tissue culture cell lines).

Also included in the invention are compositions and uses based on the mixture of THP with stabilizers well known to those skilled in the art and other additives. These compounds may be present to inhibit decay, inhibit oxidation, prevent discoloration, inhibit microbial growth, stabilize emulsions and so forth.

Also included in the invention are compositions based on THPs suitable for depressing the freezing point or inhibiting freezing in non-organic systems, such as for use in deicing treatments.

It is understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and scope of the appended claims.

EXAMPLES

The following examples are offered to illustrate, but not to limit the claimed invention.

Example 1: THP from *Tenebrio molitor* larvae

The following example details the isolation of THP from the hemolymph of *Tenebrio molitor*. *Tenebrio molitor* larvae were reared as previously described (see, Graham, *et al.*, *Insect Biochem. Molec. Biol.* 26:127 (1996)) but in darkness. Hemolymph (600 μ L) was obtained from exudates at severed prolegs of 100 presumed final instar larvae and diluted 1:1 in ice-cold hemolymph buffer (50 mM Tris-HCl (pH 8.0), 150 mM NaCl and 1 mM phenylthiocarbamide).

Diluted hemolymph (1.2 mL) was loaded onto an S-100 SEPHACRYL® (Pharmacia) column (92 cm x 1.6 cm) and eluted with hemolymph buffer without phenylthiocarbamide. Selected active fractions were combined and chromatographed by reversed-phase HPLC on a C18 analytical column (Vidac), using a gradient of 0.4% acetonitrile/min in 0.05% trifluoroacetic acid. Fractions were lyophilized and resuspended in 50 μ L of 0.1 M NH_4HCO_3 (pH 8.0) for TH measurements following standard procedures (see, Chakrabarty & Hew, *Eur. J. Biochem.* 202:1057 (1991)). Ice crystal morphology was photographed using black and white film, and crystal *c* and *a* axes were identified using cross-polarized light (Hobbs, *ICE PHYSICS*, Clarendon Press, London (1974)). Active fractions were resolved by 15% SDS-PAGE and stained using the Silver Stain Plus Kit (Bio-Rad).

Approximate molecular weights were determined by MALDI mass spectrometry. Two active, well-resolved HPLC fractions were subjected to amino acid analysis, N-terminal sequencing and electrospray ionization mass spectrometry. One fraction was reduced, alkylated, and cut with endoprotease Lys-C. Following separation of the cleavage products by reversed-phase HPLC, the best-resolved fragment was sequenced by automated Edman degradation.

When diluted larval *Tenebrio* hemolymph was fractionated by gel exclusion chromatography, there were two overlapping peaks of TH activity with apparent molecular

weights of approximately 19 and 17kD (Figure 1). When the leading peak was analyzed by HPLC, active proteins eluted between 16% and 22% acetonitrile (Figure 2) and the more abundant but inactive proteins eluted later in the gradient. Mass spectrometry (Figure 3) indicated that the inactive proteins (12.2 kDa to 12.4 kDa) migrated on SDS-PAGE according to their molecular mass, while the smaller TH proteins (7.3 kDa to 10.7 kDa) migrated anomalously as diffuse bands with apparent molecular weights ranging from 22 to 30 kDa. Fractions with the highest TH activity, corresponding to HPLC fractions 26 and 27 (Fig. 2), were analyzed for their amino acid content. The compositions of the two fractions were similar and both were rich in Thr and other amino acids with short side chains (Gly, Ala, Ser). Overall, the proteins were moderately hydrophilic (Wishard, *et al.*, *Comput. Appl. Biosci.* 10:121 (1994)). The TH activities of THP26 (fraction 26) at 55 $\mu\text{g/mL}$ and of THP27 at 21 $\mu\text{g/mL}$ were 1.6°C and 1.1°C, respectively, close to the maximum values obtained with 10-30 mg/mL of fish AFPs (Davies & Hew, *FASEB J.* 4:2460 (1990)).

Both THP26 and THP27 were N-terminally blocked. An internal fragment from THP26 released by endoproteinase Lys-C digestion was sequenced, and its amino acid content, including 7 Thr out of 20 residues, was consistent with the overall amino acid composition. Degenerate primers to this sequence were used in conjunction with vector primers to generate PCR products for screening a *Tenebrio* cDNA library.

Example 2: cDNA Library Screening for THP-encoding Genes

To isolate other isoforms of the THP of this invention, oligonucleotides for probes (SEQ ID NOs: 2 and 5), PCR primers (SEQ ID NOs: 6 and 7) and sequencing primers (SEQ ID NOs: 8 and 9) were designed based on the consensus sequence determined from sequencing TH positive clones (YL-1-4).

Aliquots of a *Tenebrio molitor* larval fat body λ -Zap cDNA library (see, Graham, *et al.*, *Insect Biochem. Molec. Biol.* 26:127 (1996)) were screened with the nucleic acid sequence of YL-1 from the 5' end to the stop codon (SEQ ID NO: 16). Approximately 1×10^5 plaques were screened at moderate stringency following standard methodologies using the sequence listed above. Isolated positive plaques were subjected to *in vivo* excision using R408 helper phage (Stratagene) as per manufacturer's instructions.

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The double-stranded DNA obtained was purified and sequenced as above using the vector primers T7 and T3 as well as SEQ ID NOs:8 and 9.

The conceptual translations from the nucleic acid sequencing matched the sequenced peptide fragment at up to 18 of the 20 residues. The first 28 amino acids represented a secretory signal peptide with Cys at the -3 and -1 positions (von Heijne, *Nucl. Acids Res.* **14**:4683 (1986)). The N-terminal amino acid of three of the variants was predicted to be Gln. This was consistent with N-terminal blockage in which the N-terminal Gln was converted to pyroglutamate by cyclization. The cleavage site of the fourth variant was not clearly predicted.

Example 3: Subcloning and Protein Expression

PCR linker-primers (SEQ ID NO 6 and 7) were designed to amplify the THP coding region and to introduce a Met codon before the presumed N-terminal residue of the mature protein. The resulting fragment was ligated into the pET-20b(+) vector (Novagen) and transformed into *E. coli* BL21(DE3). Expression was induced using isopropyl β -D-thiogalactopyranoside, cells were harvested by centrifugation, and sonicated in 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride. Cleared supernatant was loaded onto a G75 gel exclusion chromatography column. Active fractions were pooled, lyophilized, resuspended in 50 mM Tris-HCl (pH 8.0), and dialyzed overnight against the same buffer.

Example 4: Functional Activity from Cloned THP

To prove that the cloned sequence codes for a THP, the shortest of the four cDNAs with a conventional signal cleavage site (YL-2, Fig. 6) was expressed in *E. coli*. TH activity was detected in the supernate of the cell lysate, indicating that some of the protein was able to fold well enough to display activity. Partially purified recombinant THP showed 5.3°C of TH and was indistinguishable in its properties from the THP in *Tenebrio* hemolymph. Its activity was eliminated by reduction but was unaffected by chelation. Also, ice crystals formed in the presence of dilute hemolymph (Fig. 4-I) and recombinant protein were identical (Figures 4-II). These observations suggest that this THP and its isoforms can account for all the TH activity in the insect. Moreover, extensive analysis of

hemolymph and total extracts have shown no evidence of THP that are unrelated to these isoforms.

It is understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and scope of the appended claims. All publications, patents, and patent applications cited herein are hereby incorporated by reference for all purposes.